IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
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Søren Mouritsen et al.	)	Group Art Unit: 1644
	)	
Application No.: 08/955,373	)	Examiner: SCHWADRON, Ronald B.
	)	
Filed: October 21, 1997	)	
	)	
For: INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN T-CELL EPITOPES	)	Confirmation No.: 7254

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Fatema Legrand, do hereby make the following declaration:

1. I received a B.S. in Biological Science from the University of California, Los Angeles, in August 1994, an M.S. in Biological Science from Harvard University in March 1998, and completed a Ph.D. in Comparative Pathology with a Designated Emphasis in Biotechnology from the University of California, Davis in December 2001. The focus of my doctoral dissertation was the development of strategies for enhancing the safety and efficacy of recombinant vaccinia virus vaccines.

2. Since completing my Ph.D. in 2001, I have held a series of research positions of increasing responsibility, first in academic research laboratories, and subsequently in the biotechnology industry. My research has focused in the areas of vaccines and immunology, including immune monitoring and phenotypic analysis, in which I have published ten papers in peer-reviewed scientific journals. I am currently a Senior Scientist at BN ImmunoTherapeutics, Inc.

("BNIT"), a position I have held since January 2009. I have been with BNIT since August 2006. A copy of my Curriculum Vita and list of publications is attached to this Declaration as Exhibit 1.

3. I have read U.S. Patent Application No. 08/955,373 ("the '373 application"), including the claims as amended, and the Office Action issued February 17, 2011 ("Office Action"), by Examiner Schwadron. I understand that all pending claims have been rejected under 35 U.S.C. § 103(a) as allegedly obvious in view of a number of references, including International Publication No. WO 1992/005192 ("Russell-Jones"), US Patent No. 5,716,596 ("Dean"), and US Patent No. 5,969,109 ("Bona").

4. I understand that an obviousness rejection can be overcome by evidence showing that the claimed invention yields unexpectedly improved properties compared to the prior art, as has been argued in response to a previous Office Action, based upon data included in the patent specification showing that administration of a self-protein analogue modified to include a single heterologous T-cell epitope rapidly induced a strong antibody response that was not MHC restricted. US Application No. 08/955,373, at Example 3. In this case, I understand that the Examiner has asserted that the evidence of unexpected results included in the specification is not 'commensurate in scope' with the claimed invention because the claims encompass methods of treating humans, while the experiments reported in the specification were performed in mice. Office Action, section 8, page 8. Finally, I understand that such evidence need only be reasonably commensurate in scope with the claimed invention, meaning it is sufficient to establish a reasonable correlation between the evidence and the entire scope of the claim, when viewed by a skilled practitioner.

5. While it is true that the data reported in the '373 application was obtained in mice, I note that the mouse immune system is a well-established model for studying the human immune system. Indeed, preclinical studies of potential human therapeutics—immunomodulatory and

otherwise—are routinely conducted in a variety of mouse strains. Promising results obtained in mice frequently lead to the eventual conduct of clinical trials in humans. In fact, much of the preclinical work leading up to the two Phase I clinical trials described below was performed in a transgenic mouse model of HER-2-overexpressing breast cancer.

6. I also note, however, that preclinical studies generally examine a much wider range of biological responses to a particular therapeutic strategy than can be monitored in even a small clinical trial conducted in human patients, primarily for reasons of safety and cost. For example, the clinical trials described below did not collect information regarding MHC haplotype of participating patients. Furthermore, it is often impossible to replicate experimental conditions from preclinical studies in a clinical setting because patients enrolled in clinical trials are generally quite sick, and have in most cases been undergoing rigorous treatment with cytotoxic and immunosuppressive chemotherapeutics for some time. That can present a significant challenge for trials of immunotherapies in the treatment of cancer in particular, since many approved first-line chemotherapeutics have known immunosuppressive effects.

Treatment with MVA-BN®-HER2 was able to break tolerance against HER2 in metastatic HER2-overexpressing breast cancer.

7. MVA-BN®-HER2 is a candidate breast cancer immunotherapy product comprising a highly attenuated vaccinia virus, MVA-BN®, engineered to encode a modified form of the HER-2 protein. HER-2 is overexpressed in 20-30% of human breast cancers. MVA-BN®-HER2 encodes a modified form of the HER-2 protein ("HER2") containing the extracellular domain of HER2 without its intracellular cell-signaling domain that has been further modified to substitute two universal T-cell epitopes from tetanus toxin for amino acid sequences of the same length in the HER2 protein. The T-cell epitopes were included to facilitate the stimulation of an immune

response to HER2, a self-protein, which requires the 'breaking' of immune tolerance (*i.e.*, the ability to distinguish self proteins from non-self proteins).

8. Preclinical data have demonstrated MVA-BN®-HER2 to be safe and immunogenic, inducing strong antitumor activity against HER2-expressing tumors. Previous immunological evaluation of MVA-BN®-HER2-treated patient samples revealed that treatment was able to break tolerance against HER2 in a metastatic setting, inducing a humoral and/or a T-cell response in greater than 66% of the patients. Specifically, anti-HER2 antibodies were detected in 52% of patients tested and T-cell responses were boosted in 63% of patients.

9. The BNIT-BR-001 and BNIT-BR-002 Phase I trials were both fixed-dose, single arm trials conducted under an Investigational New Drug Application approved by the US Food & Drug Administration. The trials evaluated the safety and biological activity of a fixed dose of MVA-BN®-HER2 alone and in combination with chemotherapy, with or without Herceptin, for the treatment of patients with HER2-positive metastatic breast cancer. BNIT-BR-001 evaluated treatment with MVA-BN®-HER2 following first- or second-line chemotherapy (Cohort 1) alone or in combination with single-agent taxane chemotherapy (Cohort 2), while BNIT-BR-002 evaluated treatment with MVA-BN®-HER2 following first- or second-line chemotherapy. Patients were previously screened for HER2-overexpressing cancer cells.

10. Patients received 1×10^8 $TCID_{50}$ (50% tissue culture infective dose; *i.e.*, the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells) of MVA-BN®-HER2 by three consecutive subcutaneous injections administered at three week intervals. All patients from BR-001 and BR-002 were allowed to receive concurrent standard Herceptin® treatment, which was administered weekly according to the manufacturer's instructions. Patients from Cohort 2 of BR-001 received concurrent standard Taxotere® (docetaxel)

treatment administered every three weeks according to the manufacturer's instructions. Taxotere® was given one week after or two weeks before MVA-BN®-HER2 treatments.

11. Peripheral blood samples for immune analysis were collected before treatment started (pre-treatment time-point) and two weeks following each injection (post-treatment time-points). In addition, three- and six-month long-term follow up ("LTFU") samples were acquired for patients who did not manifest clinical progression. For all time-points, patient samples were drawn at the clinical trial sites and consisted of ten sodium heparin tubes, one serum separator tube, and one PaxGene tube. The clinical sites for BR-001 were located in Eastern Europe (seven sites in Serbia and Poland); samples for this study were sent overnight at ambient temperature to a central laboratory (Thymed Inc.; Frankfurt, Germany) for processing. The clinical sites for BR-002 were located in Berkeley and Stanford, CA; samples for this study were delivered to BNIT via same-day courier service. Peripheral blood mononuclear cells ("PBMC") were prepared from whole blood using Ficoll-Paque density gradient centrifugation by standard procedures. PBMC were used in assays described below and/or cryo-preserved according to standard methods.

12. Biological activity of MVA-BN®-HER2 was confirmed by measuring the humoral and/or cellular immune responses directed against HER2. Humoral immune responses were detected by enzyme-linked immunosorbent assay ("ELISA"). Cellular immune responses were detected by enzyme-linked immunosorbent spot assay ("ELISpot").

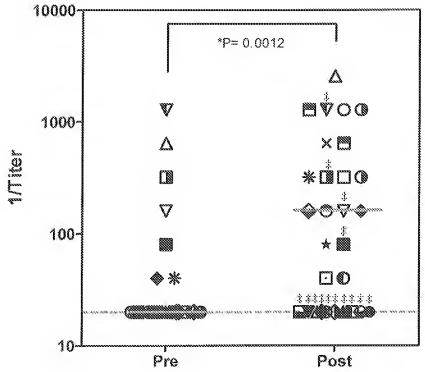
Induction of HER2- and MVA-specific humoral responses in MVA-BN®-HER2-treated individuals

13. HER2-specific and MVA-specific humoral immune responses were monitored for both BR-001 and BR-002 clinical trials by ELISA and are expressed as an antibody titer. ELISA plates (Thermo Electron, Waltham, MA) were coated with recombinant Human HER2 ECD 509 Flag or HER2 ECD 623 Flag protein diluted in coating buffer (200mM Na_2CO_3, pH 9.6) at 0.75

μg/mL or 2 μg/mL, respectively for one hour at room temperature. Plates were then washed with PBS/0.05% Tween® 20 by hand and subsequently blocked for 1 hour with PBS/0.05% Tween® 20 or SuperBlock™ (Pierce, Rockford, IL). Plates were washed with PBS/0.05% Tween® 20 before adding serial dilutions of patient sera in PBS/0.05% Tween® 20 or 10% SuperBlock™ in triplicate. Plates were incubated for one hour at room temperature then washed as above, prior to adding the detecting antibody (anti-human IgG-HRP) diluted in PBS/0.05% Tween® 20 or 10% SuperBlock™. Plates were incubated for one hour at room temperature, washed, and bound antibodies were detected with the TMB chromogenic substrate (Calbiochem, Gibbstown, NJ). The colorimetric reaction was stopped with 0.5M H_2SO_4. Absorbance at 450 nm was measured using a Multiskan Spectrum plate reader (Thermo Electronics, Waltham, MA). Titers were calculated as the last reciprocal of the highest dilution that resulted in a signal at least two fold over background. Background was established from a control sample of healthy human serum. Acceptance criteria for titer determination required statistical significance between test and control samples. Finally, vaccine-mediated effects were measured when there were no response at baseline (pre-treatment time-point) and a positive response at any post-treatment time-point or when at least a four-fold increase in titers between any time-points was detected.

14. Anti-HER2 antibody responses were evaluated by ELISA using a truncated form of the HER2 extracellular domain ("ECD") lacking domain IV, which contains the antigenic determinant recognized by Herceptin. Eighty percent of patients received Herceptin treatment in combination with MVA-BN®-HER2; use of this truncated form avoided measuring the possible interference of Herceptin in patient sera while detecting vaccine-induced anti-HER2 antibodies. Pre-treatment and peak post-treatment titers for 29 samples tested with the truncated form of the HER2 ECD are shown in Figure 1 below.

15. Figure 1:



Anti-HER2 antibody responses were detected in 15 out of 29 patients evaluated. Pre- and post-treatment sera were used for anti-HER2 antibody determination by ELISA. Post-treatment values represent peak post-treatment titers (highest serum serial dilution for which OD values are 2-fold above background). Samples not meeting ELISA acceptance criteria for anti-HER2 positivity are indicated with the symbol ‡. Median response in the pre- and post-treatment response groups is denoted with a gray horizontal bar.

16. Vaccine-induced HER2-specific antibody responses were detected in 15 out of 29 patients (>50% response rate); subsequent analysis using a flow cytometry-based assay to characterize anti-HER2 antibody binding to HER2 expressing cells revealed responses in 2 additional patients having undetectable anti-HER-2 IgG ELISA titers (data not shown). Antibody titers ranged between 40 and 2560. At least two treatments were required to reach peak titers; however, for the majority of responders (9 out of 15, or 60%) peak titers were reached after three treatments (data not shown). At long-term follow-up, HER2 antibody titers returned to baseline values after treatment cessation. The difference in titers in the pre- and post-treatment samples depicted in Figure 1 is highly statistically significant, supporting the conclusion that the increase in anti-HER2 titers observed is mediated by MVA-BN®-HER2 treatment. There was no obvious difference in response rates, kinetic and titer values between patients of Cohort 1 (6 responders out of 10 patients tested) and Cohort 2 (5 responders out of 10 patients tested) of BR-001, and BR-002 (4 responders out of 9 patients tested). Overall, seven patients had pre-existing antibody responses to HER2. This status did not significantly change the response rate. Indeed, three of these seven patients (07-057, 07-077, and 07-064) responded to MVA-BN®-HER2 with a >4-fold increased titer. Titers in samples from the remaining four patients with pre-existing anti-HER2 responses (07-065, 07-063, 07-058, and 08-003) did not augment with treatment.

17. Notably, responders and non-responders with pre-existing anti-HER2 antibody titers were evenly distributed in BR-002 and Cohort 1 of BR-001. However, no pre-existing responses were detected in samples from patients of Cohort 2 of BR-001. In terms of titer value, the strongest anti-HER2 antibody response was detected in patient 07-057 enrolled in the BR-002 trial. This patient had a pre-existing antibody response to HER2 (titer of 640 at baseline) and was treated concurrently with Herceptin. MVA-BN®-HER2 treatment stimulated the antibody response (peak

post-treatment titer of 2580) in this patient despite a decrease in T- and B-cell counts and a low NK cell count (data not shown).

18. As expected, most patients (27 out of 30 or 90%) responded to MVA-BN®-HER2 treatment by mounting or boosting responses to the vector (data not shown). Approximately one-third of the patients (9 out of 30) entered the study with a pre-existing MVA antibody titer. Of these, 7 out of 9 patients demonstrated a vaccine-induced boost in their anti-MVA titer post-vaccination. Titer variations were modest during treatment for most responders. Of the 24 patients continuing into the 3- and 6-month long-term follow-up period, 12 patients maintained the augmented anti-MVA antibody levels after the completion of treatment (data not shown).

19. Overall these data indicate that despite the immune-compromised status of the patients in these trials, MVA-BN®-HER2 was biologically active, inducing an antibody response against the HER2 transgene product in addition to the expected anti-vector responses. Importantly, pre-existing and *de novo* response to the vector did not appear to affect the induction of anti-HER2 antibody responses.

Induction of HER2- and MVA-specific T-cell responses in MVA-BN®-HER2-treated individuals

20. HER2-specific and MVA-specific cellular immune responses were monitored for both BR-001 and BR-002 clinical trials by ELISpot and are expressed as the number of spot-forming cells ("SFC"). Membranes of Millipore Multiscreen 96-well filtration plates (MSIPS4510) were hydrated with 35% ethanol. Ethanol was discarded immediately and plates were washed with PBS under sterile conditions. Plates were coated with anti-human IFN-γ Mab 1D1K capture antibody (MabTech, Sweden) at 10 μg/mL and incubated overnight at 4°C. The coating antibody was discarded, plates were washed with PBS, blocked with MATIS medium supplemented with 10% human serum for at least 60 minutes at room temperature and subsequently washed with PBS.

PBMC were added alone and stimulated with SEB (20 ng/mL), MVA virus (MOI 10 and 2), HER2 ECD and HER2 ICD overlapping peptide libraries ("OPL") (0.2 and 1 μM), HIV Gag OPL (1 μM), and CEF peptide pool (2 μg/mL). After stimulator proteins, peptides or MVA virus were added to the wells, plates were incubated at 37°C in a 5% CO_2 incubator for approximately 40 hours. Cells were then discarded and the wells were washed with PBS, and subsequently with PBS/0.05% Tween® 20. Biotin-conjugated monoclonal anti-IFN-γ antibody Mab 7B61 (MabTech, Sweden) was filtered and added to all wells. Plates were incubated for one hour at room temperature and then washed with PBS/0.05% Tween® 20 before the addition of Streptavidin-Alkaline Phosphatase (BD Pharmingen, San Diego, CA). After one-hour incubation at room temperature, the plates were washed with PBS/0.05% Tween® 20 and subsequently the backing was removed and the plate was soaked in PBS/0.05% Tween® 20 for 1 hour. The plate was developed in the dark with the Vector Blue Substrate (Alkaline Phosphatase Substrate Kit III, Vector Lab Inc., Burlingame, CA) for 20 minutes. The wells were washed gently and thoroughly with tap water to remove the substrate. Plates were scanned and spots enumerated using the CTL ImmunoSpot S3B Analyzer and ImmunoSpot Profession DC software version 5.0 (Cellular Technology Ltd., Cleveland OH). Each stimulation was performed in quadruplicate using 2×10^5 cells per well. Response evaluation was performed if at least 10 spots were counted in test wells, and a statistically significant difference in the number of spots between a test and control wells was detected. When less than 50 spots were counted in test wells, a response was deemed positive when the average number of spots in test wells was at least 2 fold higher than in the control wells. When more than 50 spots were detected in test wells, a response was deemed positive when the average number of spots in test wells was greater than the highest of the following values: control wells mean + 2 SD; control wells mean + control well mean/2 when the SD is less than 20% in the control wells. Vaccine-mediated effects

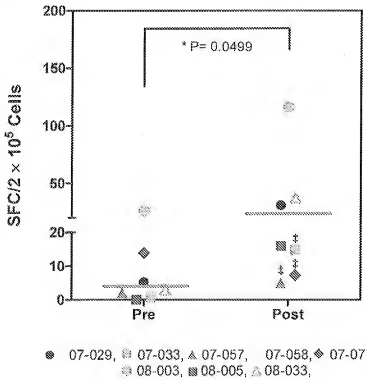
were detected when there was no response at baseline (pre-treatment time-point) and a positive response at any post-treatment time-point. For patients with pre-existing responses (*i.e.*, a positive response was detected at baseline), vaccine-mediated effects were detected when there was a significant increase (~4-fold) of spot number between baseline and post-treatment time-points. The consistency of responses to CEF peptide was used as an additional acceptance criterion to evaluate potential vaccine-induced effects.

21. Cellular immune responses were evaluated by ELISpot for eight of the nine BR-002 patients at each time-point throughout the course of the clinical trial. The lack of a fresh baseline PBMC sample rendered patient 08-024 non-evaluable for T cell analysis. All assays were performed on freshly isolated cells measuring interferon-γ (IFN-γ) secretion upon stimulation with either HER2 ECD protein (ECD-pro), a peptide library derived from HER2 ECD protein sequence (ECD-pep), a peptide library derived from HER2 ICD protein sequence (ICD-pep), tetanus toxoid peptides (TT), a mixture of peptides from cytomegalovirus ("CMV"), Epstein-Barr virus ("EBV") and influenza virus ("CEF"), or MVA. CEF stimulation was used to normalize T cell responses detected at different assay dates. Positive responses to the CEF peptide pool were detected in 7/8 patients (data not shown). CEF-specific responses in individual patients remained relatively consistent throughout the trial, with only 2 patients showing a greater variation in the CEF response after vaccination (data not shown).

22. Vaccine-induced HER2-specific T-cell responses (*de-novo* or an increase over pre-existing response) to HER2 ECD were detected in five out of eight patients (63%) upon stimulation with at least one HER2 reagent (ECD-pro, ECD-pep, and/or ICD-pep). The fact that 63% of patients displayed vaccine-induced HER2-specific T-cell responses indicates immune activation of a broad population and substantiates the preclinical observation that MVA-BN®-HER2 responses are

not MHC-restricted, since the most commonly occurring human MHC alleles appear at frequencies ranging from 29% to ~46%, depending on the population examined. *See* Exhibit 2, at Table 2; *see also* Exhibit 3.[1] Peak responses pre- and post-treatment observed with the HER2 ECD reagent are shown in Figure 2 below. Of those demonstrating a T cell response, two had pre-existing anti-HER2 T-cells that were significantly stimulated following treatment. However, in general the responses were modest (50-100 spots per 10^6 PBMC). The kinetics of the response varied, with some patients having peak responses after 1 treatment and others after 2 or 3 (data not shown). At long-term follow-up, post treatment, HER2-specific T cell levels returned to baseline values (data not shown).

23. Figure 2:

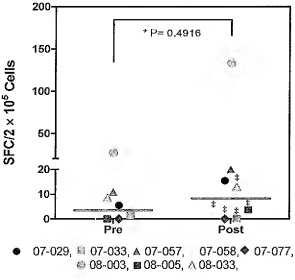


1 Table 2 of Exhibit 2 lists frequencies of the thirty most common human MHC alleles in four different populations; Exhibit 2 is available online at http://www.profimmune.com/ecommerce/page.php?page=MHC_alleles. The data was generated with a progam called HLAMatchmaker, a computer program which Exhibit 3 describes in considerable detail.

PBMC were re-stimulated *in vitro* with HER2 ECD for the induction of IFNγ secretion. Background SFC (Media/HIV Gag OPL) was subtracted for all data points; post HER- ECD values with the background subtracted represent peak post-treatment data upon re-stimulation with either an HER2 ECD OPL (1 μM) or HER2 ECD protein (10μg/mL). Samples not meeting ELISPOT acceptance criteria for anti-HER2 positivity are indicated with the symbol ‡. Median response in the pre- and post-treatment response groups is denoted with a gray horizontal bar.

24. Vaccine-induced HER2-specific T-cell responses (*de-novo* or an increase over pre-existing response) to HER2 ICD were detected in three out of eight patients evaluated upon stimulation with the HER2 ICD overlapping peptide library (ICDpep). Peak responses pre- and post-treatment observed with the HER2 ICD reagent are shown in Figure 3. Of the patients demonstrating a T cell response, two had a pre-existing HER2 ICD response that was significantly augmented and one showed a *de-novo* response to HER2 ICD. Induction of anti-HER2 ICD T-cell responses in the latter suggest possible epitope spreading to non-transgene determinants.

25. Figure 3:



PBMC were re-stimulated *in vitro* with HER2 ICD for IFNγ secretion. Background SFC (Media/HIV Gag OPL) was subtracted for all data points; post HER2 ICD response with the background subtracted represent peak post-treatment data upon re-stimulation with HER2 ICD OPL (1 μM). Samples not meeting ELISPOT acceptance criteria for anti-HER2 positivity are indicated with the symbol ‡. Median response in the Pre and Post response groups is denoted with a gray horizontal bar.

26. Strong MVA T cell responses were detected in 7 out of 8 patients tested prior to treatment that were all boosted following MVA-BN®-HER2 treatment (data not shown). The only patient without detectable pre-existing anti-MVA T cells (07-033) developed a *de-novo* response to the vector upon treatment (data not shown). As with HER-2 ECD and ICD, the kinetics of the MVA T cell responses varied, with responses reaching their peak after 1, 2 or 3 vaccinations (data not shown). At long-term follow-up, post treatment, MVA-specific T cell levels returned to baseline values (data not shown). These data corroborate the antibody data described above, as they confirm the potency of MVA-BN®-HER2 at inducing immune responses to both the HER2 transgene product and vector. As with the antibody responses, pre-existing and *de novo* responses to the vector did not appear to affect the induction of anti-HER2 T-cell responses.

27. In summary, the data presented above shows that MVA-BN®-HER2 is immunogenic in human patients having HER2-overexpressing breast cancer. Anti-MVA antibody responses were detected in 27 out of 30 patients tested (90% response rate), while anti-MVA T cell responses were detected in 8 out of 8 patients tested (100% response rate). Most importantly, MVA-BN®-HER2 immunogenicity was also established based on the induction of immune responses to HER2, a self tumor antigen, a variant form of which is expressed by the viral vector. Overall, anti-HER2 antibody and/or T-cell responses were detected in 19 out of 29 patients tested (66% response rate).

Hence, MVA-BN® HER2 treatment was able to break tolerance against HER2 in a majority of metastatic breast cancer patients, which validates this compound as a vaccine candidate for cancer immunotherapy and confirms the findings of the preclinical studies reported in the '373 application.

28. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: June 17, 2011

By: _____
Fatema Legrand, Ph.D.

EXHIBIT 1

Fatema A. Legrand
2425 Garcia Avenue
Mountain View, CA 94043

EDUCATION

Ph.D., University of California, Davis, Comparative Pathology with a Designated Emphasis in Biotechnology, December 2001. Dissertation: Enhancing the safety and efficacy of recombinant vaccinia virus vaccines.

Master of Science, Harvard University, Biological Science, March 1998. Thesis: The role of viral anti-inflammatory genes and lymphokines on the immune response.

Bachelor of Science, University of California, Los Angeles, Biological Science, August 1994.

INDUSTRY EXPERIENCE

Senior Scientist, BN ImmunoTherapeutics (a subsidiary of Bavarian-Nordic), January 2009-Present

Human immunology group leader, supervising 7 personnel including 5 research associates, 1 scientist, and 1 QA specialist. Coordinate the scientific and logistical facets of the clinical trial immune monitoring for phase I, II and III recombinant modified vaccinia Ankara (MVA) based cancer immunotherapy programs. Supervise development of QC product characterization and release assays under a GMP compliant environment.

Research Scientist II, BN ImmunoTherapeutics (a subsidiary of Bavarian-Nordic), August 2006-Decmber 2008

Lead the clinical immune monitoring group comprised of a team of 4 Research Associates and 1 Clinical Research Associate. To implement and coordinate a clinical immune monitoring program for two recombinant modified vaccinia Ankara (MVA) based cancer immunotherapies in international clinical trials. Duties include developing cutting-edge cell-based assays such as flow cytometric based phenotypic analysis of peripheral blood subsets including T regulatory cells, Enzyme-Linked Immunospot (ELISPOT) assay, Intracellular Cytokine Staining (ICS), flow cytometric based proliferation assay, flow cytometric based cytotoxicity assays, multiplex cytokine analysis using the BD CBA system, and RT-PCR based measurement of circulating tumor cells. Other efforts have focused on developing multiplex assays for the measurement anti-tumor humoral immune responses as well as a peptide based B-cell epitope mapping assay. Additional responsibilities encompass the quality control of assays and reported data, troubleshooting, method validation, ensuring GLP/GCP compliance, interpretation and synthesis of study results, writing SOPs and technical reports, preparation of supporting documentation, including documents contributing to regulatory submissions, training personnel, supervising laboratory day-to-day operations and safety, and interacting with multidisciplinary project teams and CROs.

RESEARCH EXPERIENCE

Postdoctoral Research, University of California, San Francisco/Gladstone Institute of Virology and Immunology, June 2003-July 2006

Understanding the function of the infant immune system in the setting of pediatric HIV infection. Showed that exposed-uninfected infants can mount HIV-specific T-cell immune responses at high frequency and that CD4+ CD25+ T-regulatory cells may play a role in subverting infection, determined that strong CD4+ T-helper and CD8+ cytotoxic T-cell immune responses elicited against HIV viral antigens play a central role in controlling disease progression in a set of perinatally infected monozygotic twins that differ greatly in their clinical course where one twin is thriving while the other is not, elucidated the role of protease inhibitors in inhibiting cell-mediated immune responses in HIV-infected children.

Postdoctoral Research, University of California, Davis, January 2002-May 2003 Completion of doctoral research (see below) and preparation of manuscripts for publication.

Doctoral Research, University of California, Davis, September 1997-December 2001 Demonstrated that the inactivation of the vaccinia virus B13R or B22R immune-modulating gene greatly attenuates recombinant vaccinia virus vaccines without a concomitant loss of immunogenicity, showed that the co-expression of the cytokine IFN-γ substantially increases attenuation while maintaining strong humoral and cell-mediated immune responses, revealed that the cytokine IL-18, also known as IFN-γ inducing factor, can be utilized as a potent adjuvant as well as an attenuating agent, and co-developed a second-generation vaccine for rinderpest that has the potential for global eradication of the disease, and participated in the study of the HIV-1 accessory protein Nef. While this protein serves as a virulence factor in its host virus, in a large DNA virus like vaccinia virus, Nef acts as an attenuating agent.

Graduate Biotechnology Research Intern, Scios Inc., Sunnyvale, CA, April 2000-October 2000 Researched the biological role(s) of p38-gamma using an adenovirus system.

Masters Research, Harvard University, 1995-1997 Constructed recombinant viruses for the study of the immune-modulating role(s) of viral anti-inflammatory genes and cytokines.

Honors Undergraduate Research, University of California, Los Angeles, 1994-1995 Studied the role of specific T-cell receptor repertoires in controlling the murine autoimmune diseases experimental allergic encephalomyelitis and collagen-induced arthritis.

PATENTS

METHODS FOR TREATING PROSTATE CANCER WITH MVA. The invention encompasses methods for treating prostate cancer patients with a recombinant modified vaccinia Ankara (MVA) viral vector encoding genes for prostate specific antigen (PSA) and prostate acid phosphatase (PAP).

PUBLICATIONS

Fatema A. Legrand, Douglas F. Nixon, Christopher P. Loo, Erika Ono, Joan M. Chapman, Maristela Miyamoto, Ricardo S. Diaz, Amélia M.N. Santos, Regina C.M. Succi, Jacob Abadi, Michael G. Rosenberg, Maria Isabel de Moraes-Pinto, Esper G. Kallas. 2006. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants

and neonates revealed after regulatory T cell removal. PLoS ONE. 1(1): e102 doi:10.1371/journal.pone.0000102.

Fatema A. Legrand, Jacob Abadi, Kimberly A. Jordan, Miles P. Davenport, Steve G. Deeks, Glenn J. Fennelly, Andrew A. Wiznia, Douglas F. Nixon, and Michael G. Rosenberg. 2005. Partial treatment interruption of protease inhibitors augments HIV-specific immune responses in vertically infected pediatric patients. AIDS. 19(15): 1575-1585.

Kenneth S. Chan, Paulo H. Verardi, Fatema A. Legrand, and Tilahun D. Yilma. 2005. Nef from pathogenic simian immunodeficiency virus is a negative factor for vaccinia virus. Proceedings of the National Academy of Sciences, USA. 102(24): 8734-9.

Fatema A. Legrand, Paulo Verardi, Kenneth S. Chan, Yue Peng, Leslie Jones, and Tilahun D. Yilma. 2005. Recombinant Vaccinia Viruses Expressing Interferon-Gamma are Highly Attenuated and Efficacious. Proceedings of the National Academy of Sciences, USA. 102 (8): 2940-5.

Fatema A. Legrand, Paulo H. Verardi, Leslie A. Jones, Kenneth S. Chan, Yue Peng, and Tilahun D. Yilma. 2004. Induction of Potent Humoral and Cell-Mediated Immune Responses with Attenuated Serpin-Deleted Vaccinia Virus Vaccine Vectors. Journal of Virology. 78 (6): 2770-2779.

Tilahun D. Yilma, Fatema H. Aziz, Shabbir Ahmad, Leslie A. Jones, Rosemary N. Ngotho, Henry M. Wamwayi, Berhanu Beyene, Mebratu G. Yesus, Berhe G. Egziabher, Martiam Diop, Joseph Sarr, and Paulo

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HONORS/DISTINCTIONS/FELLOWSHIPS

University of California President's Postdoctoral Fellowship 2004-2006 National Institutes of Health Loan Repayment Postdoctoral Fellowship 2004-2006

Graduated-Summa Cum Laude, University of California, Davis, 2001 University of California, Davis Biotech Program Fellowship 1999-2000, 2000-2001 Floyd and Mary Schwall Fellowship-University of California, Davis, 1997-2001 Peter J. Shields Fellowship-University of California, Davis, 1997, 1998, 1999 Jastro Shields Fellowship-University of California, Davis, 1998, 1999, 2000 USDA Formula Funds-University of California, Davis (1999, 2000, 2001)

Graduated Top of Class, Summa Cum Laude, Harvard University, 1998 Thomas Small Prize-To Top Student in the Graduating Class, Harvard University, 1998 Deans List-Harvard University, Fall and Spring semesters, 1996, 1997

Dean's List-University of California, Los Angeles, 1992, 1993, 1994 Honors thesis-Collagen-Induced Murine Arthritis-University of California, Los Angeles, 1994

TEACHING EXPERIENCE

Teaching Assistant-1998, 2000 Lectured, demonstrated new techniques, and assisted students in a veterinary virology laboratory class. Dr. Tilahun D. Yilma, Department of Veterinary Pathology, Microbiology and Immunology

ACADEMIC SERVICE

Gladstone's Minority and Youth Outreach Program-Mentor, University of California, San Francisco/Gladstone Institutes, 2005-2006 Expand Your Horizon's Program-Mentor-inspiring teenage girls to pursue science, University of California, Davis, 2000 Graduate Student Association-Graduate Student Representative for the Comparative Pathology Graduate Group, University of California, Davis (1997-1998) National Science Foundation High School Student Summer Program-Mentor, University of California, Davis, 1998 UCLA Undergraduate Science Journal-Associate Editor, University of California Los Angeles (1994-1995)

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Available upon request.

EXHIBIT 2

MHC Alleles

When planning an experiment it is important to decide which epitopes are to be studied and to know their corresponding Major Histocompatibility Complex (MHC) restriction.

When using a mouse experimental model it is important to know which MHC allele is expressed by the strain of inbred mouse to be used, Table 1.

For example, H-2 Kb MHC Pentamers can only be used with samples from C57BL/6 mice. The T cell receptors of these mice are restricted to H-2 Kb or H-2 Db for class I MHC and therefore will not interact with H-2 Kd Pentamers, for example.

When analyzing human samples it is important to know that the donor is positive for the MHC allele to be investigated. Donor samples therefore either have to be tissue typed or at least serotyped for the allele(s) of interest. Table 2 shows the frequency of the top 30 human class I alleles in four ethnic groups (Caucasian, African-American, Hispanic and Oriental). In certain disease areas and populations the immune response to some epitopes is well characterized, e.g. CMV, EBV. In these cases the published literature will assist experimental planning. The technical support team at ProImmune is happy to offer advice about epitopes and MHC alleles to be used in a given study.

Table 1

MHC alleles expressed by commonly used inbred mouse strains.

			H-2 Class I			H-2 Class II	
Strain	**Appearance**	**Haplotype**	**K**	**D**	**L**	**IA**	**IE**
Balb/c	albino	*d*	Kd	Dd	Ld	IAd	IEd
C3H/He	agouti	*k*	Kk	Dk	-	IAk	IEk
C57BL/6	black	*b*	Kb	Db	-	IAb	-
CBA	agouti	*k*	Kk	Dk	-	IAk	IEk

Table 2

Frequency of the top 30 human class I MHC alleles in the North American population.

Percentage chance of allele expressed in an individual

Top 30 expressed alleles

Allele	Caucasian	Allele	African American	Allele	Hispanic	Allele	Oriental
A*02:01	45.6%	C*04:01	29.0%	A*02:01	37.1%	A*11:01	38.4%
C*07:01	27.7%	C*07:01	25.4%	C*04:01	25.4%	A*24:02	33.7%
A*01:01	27.4%	C*06:02	23.0%	A*24:02	24.9%	C*07:02	33.3%
A*03:01	23.8%	A*02:01	22.3%	C*07:02	24.2%	C*01:02	27.7%
C*07:02	21.5%	A*23:01	20.7%	C*07:01	20.8%	A*33:03	23.3%
C*04:01	21.2%	C*02:02	19.0%	C*03:04	14.4%	C*08:01	21.6%
B*44:02	20.2%	A*03:01	18.7%	A*03:01	14.3%	C*03:04	19.9%
B*07:02	18.1%	C*07:02	18.1%	B*07:02	13.2%	A*02:01	18.1%
B*08:01	18.1%	B*53:01	18.1%	B*35:01	12.8%	B*40:01	15.2%
C*05:01	17.2%	B*07:02	15.8%	C*06:02	12.3%	C*04:01	14.0%
C*03:04	16.8%	C*16:01	15.7%	C*05:01	11.9%	B*58:01	13.3%
C*06:02	15.7%	B*15:03	13.9%	A*01:01	11.4%	B*46:01	12.7%
A*11:01	15.3%	B*58:01	13.5%	A*11:01	11.0%	B*51:01	12.4%
B*40:01	13.6%	A*68:02	12.7%	B*51:01	10.8%	C*03:02	12.0%
A*24:02	12.1%	C*17:01	11.7%	C*16:01	10.6%	B*38:02	11.4%
B*35:01	10.7%	B*45:01	10.8%	B*44:03	9.9%	A*02:07	11.0%
C*03:03	10.6%	B*42:01	10.5%	C*01:02	9.7%	B*15:01	9.4%
B*51:01	10.4%	A*30:01	10.4%	A*29:02	9.7%	A*02:06	9.3%
C*12:03	9.9%	B*35:01	10.1%	C*08:02	9.3%	C*03:03	9.2%
B*15:01	9.6%	A*01:01	10.0%	B*18:01	9.1%	B*15:02	9.1%
A*29:02	8.9%	C*03:04	9.3%	A*31:01	8.9%	A*02:03	8.8%
A*26:01	8.2%	A*30:02	9.2%	B*52:01	8.6%	B*44:03	8.6%
A*32:01	8.2%	B*08:01	8.5%	B*14:02	8.6%	C*14:02	8.4%
C*08:02	7.7%	A*34:02	8.4%	C*02:02	7.6%	B*35:01	7.2%
A*25:01	7.5%	A*74:01	8.4%	C*12:03	7.6%	C*06:02	7.0%
B*57:01	7.1%	A*33:03	8.0%	A*26:01	7.6%	B*54:01	6.9%
B*14:02	6.7%	C*18:01	7.3%	A*68:01	7.1%	B*13:01	6.6%
C*02:02	6.6%	A*29:02	7.2%	B*08:01	7.0%	B*40:02	6.3%

B*18:01	6.4%	B*44:03	6.9%	A*30:02	6.8%	B*55:02	6.3%
B*44:03	6.4%	B*49:01	6.9%	B*44:02	6.5%	A*26:01	6.0%

Data from HLA Matchmaker, http://tpis.upmc.edu/tpis/HLAMatchmaker/

(Top)

EXHIBIT 3

ELSEVIER

HLAMatchmaker: A Molecularly Based Algorithm for Histocompatibility Determination. I. Description of the Algorithm

René J. Duquesnoy

ABSTRACT: This report describes an algorithm for identifying acceptable HLA antigens for highly alloimmunized patients without the need for extensive serum screening. This algorithm is based on the concept that immunogenic epitopes are represented by amino acid triplets on exposed parts of protein sequences of human leukocyte antigen chains (HLA-A, HLA-B, and HLA-C) accessible to alloantibodies. A computer program (HLAMatchmaker) has been developed to determine class I HLA compatibility at the molecular level. It makes intralocus and interlocus comparisons of polymorphic triplets in sequence positions to determine the spectrum of non-shared triplets on donor HLA antigens. In most cases is it possible to identify certain mismatched HLA antigens that share all their polymorphic triplets with the patient's HLA antigens and could therefore, be considered fully compatible. HLAMatchmaker permits also the identification of additional mismatches that are acceptable as determined from the triplet information on HLA-typed panel cells that do not react with patient's serum.

HLAMatchmaker provides an assessment of donor-recipient HLA compatibility at the structural level and this algorithm is different from conventional methods based on the mere counting of numbers of mismatched HLA antigens or CREGs. This donor selection strategy is suitable especially for allosensitized patients in need of a compatible transplant or platelet transfusion. *Human Immunology 63, 339–352 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: histocompatibility; HLAMatchmaker; triplet; PRA; highly sensitized patients

INTRODUCTION

Transplant candidates are generally considered highly alloimmunized if their serum panel reactive antibody (PRA) activity exceeds 85%. Such patients can be successfully transplanted provided that the donor has no mismatched human leukocyte antigens (HLA) that react with the patient's alloantibodies. Serum screening against HLA-typed panels must be done to determine the overall antibody specificity spectrum so that unacceptable HLA antigen mismatches can be avoided. Many screening protocols are based on complement-dependent lymphocytoxicity determined by direct testing, such as the NIH standard and Amos modified tests [1–3], and by the more sensitive anti-human globulin (AHG) augmentation technique [4–6]. Newer methodologies for serum screening include enzymeimmunoabsorbent assays (ELISA) [7–10] and flow cytometric analysis [11–13].

Most clinical laboratories screen sera against HLA-typed panels ranging generally from 30 to 60 in size. Serum reactivity patterns are analyzed by 2×2 table statistics, such as the Chi-square test, to determine significant correlations between positive reactions and the specificity of HLA antigens in the panel. Besides the WHO-designated HLA antigens, correlations have been determined for public epitopes assigned from so-called cross-reacting groups (CREGs) of HLA antigens [14–21], and for amino acid polymorphisms defined from sequence information of HLA molecules [22–24]. Significant correlations provide an assessment of the alloantibody specificity patterns of sera and this information permits a determination whether mismatched HLA antigens of a potential donor might be acceptable or unacceptable to the patient.

CLSI Tissue Typing Laboratory, Department of Pathology, Division of Transplantation Pathology,Thomas E. Starzl Transplantation Institute, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

Address reprint requests to: Dr. René J. Duquesnoy, Pathology and Surgery, Biomedical Science Tower, Room W1552, University of Pittsburgh Medical Center, Pittsburgh, PA 15261 USA; E-mail: duquesnoyr@msx.upmc.edu.

Received December 6, 2001; revised January 20, 2002; accepted January 28, 2002.

0198-8859/02/$–see front matter
PII S0198-8859(02)00387-8

This approach works generally quite well if the patient's serum is not overly reactive and its PRA is <80%. However, the 2×2 table statistics becomes unreliable for highly reactive sera. For instance, a serum with a 96% PRA against a 50-cell panel, manifests negative reactions with only two panel cells. This number is too small for meaningful interpretations of Chi-square statistics, especially if the serum has two or more HLA-antibody specificities. This problem might be overcome by screening high PRA sera against large cell panels [17, 24], and/or perform absorption/elution studies with selected cells and then rescreen the absorbed sera and the eluates [25–28]. However, such methods for alloantibody identification are very labor intensive, and they require considerable resources not readily available in a clinical laboratory setting. Often enough, many highly sensitized patients remain on the waiting list with little prospect of a transplant because no information is available about acceptable HLA mismatches and the probability of finding a zero antigen mismatch is very low.

Another method for analyzing screening results focuses on the identification of acceptable antigen mismatches expressed by panel cells that give negative reactions with patient's serum [29–31]. It can also be applied to patients with extremely reactive sera (*i.e.*, 100% PRA by routine panel screening) if panel cells are used that are selectively mismatched for a single HLA antigen. The acceptable mismatch approach works generally quite well for patients with common HLA antigens in their phenotypes, but it requires access to a very large pool of HLA typed panel cell donors.

This report describes an alternative strategy for identifying potential donors for highly sensitized patients. HLAMatchmaker is a computer-based algorithm that focuses on the structural basis of HLA class I polymorphisms so that compatible HLA mismatches can be determined for each patient without the need for extensive serum screening.

HLAMatchmaker considers amino acid sequence polymorphisms as critical components of immunogenic epitopes that can elicit alloantibodies. Such amino acids reside in sequence positions accessible to alloantibodies, namely the α-helices and β-loops (also referred to as β-bulges or β-turns between the secondary structures) of the protein chain structure. Each HLA molecule expresses on its surface multiple amino acid defined antigenic determinants recognized by distinct alloantibodies [18, 22, 25, 32–46]. The residues in the strands of the β-pleated sheets of the peptide-binding groove are excluded from this matching algorithm because they cannot make direct contact with alloantibodies.

This HLA matching algorithm is based on comparisons of linear sequences of amino acid triplets as motifs for potentially immunogenic epitopes. Each HLA antigen represents a distinct string of polymorphic triplets and an HLA mismatch is assessed by determining the number of triplets not shared with the recipient's HLA antigens. This report describes the logistics of HLA-Matchmaker and how this algorithm permits the identification of HLA-compatible donors for highly sensitized patients without the need for extensive serum screening.

METHODS AND RESULTS

Amino Acid Triplet Polymorphisms of Antibody-Accessible Sites of HLA Class I Molecules

The assignment of antibody-accessible positions is based on the detailed descriptions of the crystalline structure of various HLA class I molecules (HLA-A2, -A68, and -B27) [47–50]. The definition of the repertoire of triplets considers published amino acid sequences of serologically defined HLA antigens (see the IMGT/HLA database at http://www.anthonynolan.com/HIG/index.html).

Table 1 represents the total repertoire of polymorphic triplets in the antibody-accessible positions of amino acid sequences of serologically defined HLA-A, HLA-B, and HLA-C antigens. This list resulted from a comparative analysis of the amino acid sequences of molecular equivalents of serologically defined HLA antigens. Each triplet is designated by its amino acid composition around a given position in the amino acid sequence. Amino acid residues are marked with the standard letter code; an uppercase letter corresponds to the residue in the numbered position, whereas the lowercase letters describe the nearest neighboring residues. For instance, the triplet a65rNm represents an asparagine residue (N) in position 65 with arginine (r) in position 64 and methionine (m) in position 66 of the HLA-A chain. Many triplets are marked with one or two residues because their neighboring residues are the same on all HLA class I chains and, therefore, they are not listed. For instance, b12aM represents an alanine residue in position 11 and a methionine residue in position 12 on HLA-B chains. The triplet b41T has a threonine in position 41 and the two neighboring monomorphic residues are not listed.

Most polymorphic triplets reside in the membrane-terminal α1 (positions 1–90) and α 2 (positions 91–182) domains of the HLA chains. The locations of the triplets on the α helices and the β loops of the molecular structure are described according to the helix (H1, H2, etc.), strand (S1, S2, etc.), and loop (S1 → S2, S2 → S3, etc.) annotations as previously reported [48]. On each domain, a very short nearly vertical helix (H1) precedes a long curved (H2 in α1) or kinked (H2a, H2b, and H3

TABLE 1 Polymorphic triplets in antibody-accessible locations on HLA-A, HLA-B, and HLA-C molecules

Location	HLA-A	HLA-B	HLA-C
α1S1 → S2	a9 F* S* T Y*	b9 D* **H** Y*	c9 D* F* S* Y*
α1S1 → S2	a12 sV	b12 sV° aO aV*	c12 sV° aV*
α1S1 → S2	a14 R	b14 R	c14 R° **W**
α1S1 → S2	a17 gR° **gS**	b17 gR	c17 gR° **sR**
α1S3 → S4	a41 A	b41 A° **T**	c41 A
α1S3 → S4	a45 **Me kMe**	b45 **Ee Te** Ge° **Ke Ma GeV**	c45 Ge
α1H2	a56 G° **R E**	b56 G	c56 G
α1H2	a62 Rn* **Qe Ee Lq** Ge*	b62 Rn* Re° Ge*	c62 Re
α1H2	a66 **rKv rNv gKv** rNm*	b66 qKy* **Qlc qls qly qlf** rNm*	c66 qKy* **gNv**
α1H2	a70 **aQs aHs**	b70 **aQa tNt aKa** aSa rQa°	c70 rQa
α1I12	a74 D* **N H iD**	b74 **D* Y**	c74 D* **aD**
α1I12	a76 **An Vd** En* Es*	b76 Es* En* **Ed** Vs° **Vg**	c76 Vs* **Vn**
α1H2	a80 **gTI** rla*	b80 rla* rNI* **rTI rTa**	c80 rNI* **rKi**
α1H2	a82 aLr* IRg°	b82 aLr* IRg° **ILr**	c82 IRg
α1H2 → α2S1	a90 D* A*	b90 A* D*	c90 A* D*
α2S1 → S2	a105 P* S	b105 P	c105 P
α2S1 → S2	a107 G° **W Grl**	b107 G	c107 G
α2S3 → S4	a127 N° **K**	b127 N	c127 **N**
α2S3 → S4	a131 R	b131 R° S	c131 R
α2S4 → H1	a138 T	b138 T	c138 T° **K**
α2H1	a142 I* **T**	b142 I	c142 I
α2H1	a144 **tKr tKh** tQr°	b144 tQr° **tQI tQr**	c144 tQr
α2H1	a147 W	b147 W° L*	c147 W° L*
α2H1	a149 **aVh aAh** aAr° **tAh**	b149 aAr	c149 aAr
α2H2a	a151 **yHa aHv aHe** aRv* **aRw aRr aHa**	b151 aRv* aRe*	c151 **aRt** aRe* **aRa**
α2H2a	a156 L* W* R* **Q**	b156 W* L* **D** R*	c156 L* R* W*
α2H2a	a158 A° **V**	b158 A° **T**	c158 A
α2H2b	a156 **R** T* **dT** E*	b163 **L*** F* T* **dL**	c163 T* E* L*
α2H2b	a166 **Dg** Iw°	b166 Ew° **Es**	c166 Ew
α2H2b	a171 V° H*	b171 V° H*	c171 Y
α2H3	a177 Er	b177 **Dt** Et° **Dk**	c177 **Kt** Et
α2H3.	a180 Q	b180 Q° **E**	c180 Q
α2H3 → α3S1	a184 dP° **dA**	b184 dP	c184 **cH cP**
α2H3 → α3S1	a186 K° **R**	b186 K	c186 K
α3S1 → S2	a193 **Av** Pi*	b193 Pi* Pv*	c193 Pv* **PI**
α3S1 → S2	a199 A	b199 A° **V**	c199 A
α3S1 → S2	a207 G° **S**	b207 G	c207 G
α3S5 strand	a246 A° **S Va**	b246 A° **E**	c246 A
α3S5 strand	a248 V	b248 V° **G**	c248 V* **Q**
α3S5 → S6	a253 Ie° **Ke Qe**	b253 Ee	c253 Ec° **Eq**

Triplets in underlined bold font are uniquely present on one (or two) HLA antigens; triplets in bold font (not underlined) are found on groups of three or more cross-reacting antigens within the same locus; triplets marked by a superscript (*) are polymorphic at one locus but monomorphic at another locus; triplets marked by an asterisk (*) are polymorphic at two or three loci.

The triple selection was based on the sequences of serologically defined HLA antigens corresponding to: A*0101 A*0201 A*0301 A*1101 A*2301 A*2402 A*2501 A*2601 A*2902 A*3001 A*3101 A*3201 A*3301 A*3402 A*3601 A*4301 A*6601 A*6801 A*6901 A*7401 A*8001 B*0702 B*0801 B*1302 B*1801 B*2705 B*3501 B*3701 B*3801 B*3906 B*4101 B*4201 B*4402 B*4501 B*4601 B*4701 B*4801 B*4901 B*5001 B*5101 B*5201 B*5301 B*5401 B*5501 B*5601 B*5701 B*5801 B*5901 B*4001 (B60) B*4002 (B61) B*1501 (B62) B*1516 (B63) B*1401 (B64) B*1402 (B65) B*6701 B*1509 (B70) B*1510 (B71) B*1503 (B72) B*1301 B*1502 (B75) B*1511 (B76) B*1513 (B77) B*7801 B*8101 B*8201 Cw*0102 Cw*0202 Cw*0302 Cw*0401 Cw*0501 Cw*0602 Cw*0701 Cw*0801. Note these alleles were selected because of their relatively high frequencies in our local population; they are used in the triplet matching examples described in this article. Although other alleles might be more frequent in different populations, they have the same triplets as those shown in this table.

in α2) helix. As depicted in Table 1, triplet polymorphisms can be found in 8 locations of the α1 helix (α1 72 2) and 12 locations in the α2 helix (α2H1, α2H2a, α2H2b and α2H3).

There are four β-loops in the α1 domain and the polymorphic triplets are defined within the following sequences: α1S1 → S2 (residues 9 – 21), α1S2 → S3 (28–31), α1S3 → S4 (37–46), and α1S4 → H1 (47–50). Similarly, the α2 domains have four β-loops: α2 S1 → S2 (residues 103–109), α2 S2 → S3 (118–121), α2 S3 → S4 (126–133), and α2 S4 → H1 (135–138). The α1 S2 → S3 and α2 S2 → S3 loops extend below the β-sheets and interact with the α3 domain and the β_2-microglobulin part of the HLA molecule, respec-

tively. These β-loops do not exhibit any triplet polymorphisms and they seem inaccessible to antibodies. The remaining β-loops extend above the β-sheet and pack against the outer faces of the α-helices and all appear to be antibody accessible. The α1H2 → α2S1 loop between the α1 and α2 domains (residues 84–94) forms a flat open structure on the top surface of HLA adjacent to the antigen-binding cleft. Triplet polymorphisms can be found in a total of 12 locations of the β-loops of the α1 and α2 domains and the interdomain loop.

The α3 domain of the HLA molecule has a fold, like an immunoglobulin constant domain and its seven β-strands reveal considerable structural homologies with β2-microglobulin and the CH3 domain of IgG [48]. There are six loops between the β-strands. The α3 domain is generally much less polymorphic than the α1 and α2 domains and triplet polymorphisms may be found in two locations of the β-loops (α3S1 → S2 and α3S5 → S6). The loop between the α2 and α3 domains has two locations where triplet polymorphism may occur.

Are there other polymorphic locations in the α3-domain that might be accessible to antibodies? The α3-domain can bind to the T-cell accessory molecule CD8 and the contact region involves the α3S3 → S4 β-loop (residues 224–228) and other residues near position 245 in the β-strand α3S5 [51, 52]. This part of the α3-domain may also serve as a contact region for immunoglobulin variable domains [48] and, therefore, might be accessible to antibodies. Whereas the α3S3 → S4 β-loop is highly conserved, the β-strand α3S5 has two locations where triplet polymorphisms may occur.

A total of 142 different polymorphic triplets have been designated to the serologically defined HLA-A, -B, and -C antigens (Table 1). Triplet polymorphisms occur at 30 locations on HLA-A chains, 27 locations on HLA-B chains, and 19 locations on HLA-C chains. The polymorphic triplets have been categorized into four groups according the potential of being recognized as non-self or self when the patient is exposed to an HLA mismatch. The first group consists of triplets that are present on one or two HLA antigens. They are depicted in bold underlined font in Table 1. Several private HLA antigens can be distinguished by unique triplets. For instance, a163dT is found only on HLA-A3 molecules and a monospecific antibody to HLA-A3 seems to react with an a163dT-based epitope. Other HLA antigens have unique triplets and their serologic splits can be distinguished with other triplets. For instance, HLA-B16 has a unique b158T triplet and its splits HLA-B38 and HLA-B39 can be distinguished by triplets b80rIa and b82aLr versus b80rNl and b82lRg, respectively. These triplets correspond to the Bw4 and Bw6 epitopes.

Some HLA antigens have two or more unique triplets in their sequence. For instance, HLA-A30 has two unique triplets: a17S and a151aRw, and it seems possible that HLA-A30 specific antibodies recognize two structurally distinct epitopes. The uncommon antigen HLA-A80 has five unique triplets and HLA-Cw7 has four unique triplets. It should be noted that many private HLA-A and HLA-B antigens do not have corresponding unique triplets in the antibody-accessible sites of their sequence.

Several triplets can be uniquely found on pairs of HLA antigens. Examples are a56R on HLA-A30+A31 and a76Es on HLA-A25+A32. The serologic cross-reactivity of these antigen pairs is well-known and it seems likely that these triplets are critical components of the antigenic epitopes recognized by cross-reacting antibodies.

The second group consists of polymorphic triplets that are shared between three or more HLA antigens encoded by the same class I locus; they are depicted in bold font in Table 1. Several of them seem to correspond with public epitopes or CREGs. For instance, the members of the CREG HLA-A2, HLA-A23, HLA-A24, HLA-A68, and HLA-A69 antigens share a127K, which may represent a distinct public epitope recognized by alloantibodies in sensitized patients. Similarly, the CREG HLA-B7, HLA-B40, and HLA-B48 antigens have a distinct b177Dk triplet.

The third group consists of triplets that are polymorphic for one class I locus but monomorphic for another class I locus; they are marked with a ° symbol in Table 1. Such triplets cannot represent immunogenic epitopes because they are always present on the patient's own HLA antigens. For instance, the a56G polymorphic triplet is found on all HLA-A molecules except HLA-A30 and HLA-A31, but the corresponding triplet at position 56 on HLA-B (b56G) or HLA-C (c56G) is monomorphic. This means that a56G is always a self-triplet and, therefore, cannot be immunogenic. Similarly, b12sV is polymorphic for HLA-B but this triplet cannot be immunogenic because all patients have the monomorphic a12sV triplet on their HLA-A molecules.

The fourth group of triplets are polymorphic for two (or all three loci) and they are marked with a * symbol in Table 1. Several of them represent well-known interlocus public epitopes. For instance, HLA-A2 and HLA-B17 share a public epitope [53, 54] that corresponds to the Ge triplet in position 62. This triplet is called a62Ge on HLA-A2 and b62Ge on HLA-B17 chains and monoclonal antibodies specific for HLA-A2+B17 recognize an epitope that corresponds to 62Ge [55]. Another example is the interlocus public epitope shared between the Bw4 group of HLA-B antigens and the A locus antigens HLA-A23, HLA-A24, HLA-A25, and HLA-A32 [56, 57]. This epitope corresponds to sequences marked by

TABLE 2 Summary of HLA-A, B, and C triplet polymorphisms

Triplet polymorphisms	HLA-A	HLA-B	HLA-C	Total number of unique triplets
1. Triplets on one or two HLA antigens encoded by the same locus	25	11	8	44
2. Triplets shared between three or more HLA antigens encoded by the same locus	24	24	8	56
3. Polymorphic triplets that are monomorphic for another locus (loci)	17	17	7	29
4. Polymorphic triplets that are also polymorphic for another class I locus	21	28	20	33
Total number of triplets:	87	80	42	142

Abbreviation: HLA = human leukocyte antigen.

the 76En, 80rIa, and 82aLr triplets. The HLAMatchmaker algorithm includes interlocus comparisons between polymorphic triplets at the different HLA loci to determine whether a triplet on a mismatched HLA molecule must be considered as non-self. For instance, patients who type for HLA-B17 should not recognize a62G on an HLA-A2 mismatch as non-self because HLA-B17 molecules carry the same triplet b62G in the same sequence position as a62G.

Table 2 illustrates an enumeration of the four groups of polymorphic triplets on HLA-A, HLA-B, and HLA-C molecules. In the overall repertoire of 142 polymorphic triplets, a total of 29 triplets (group 3) cannot be considered as immunogenic, whereas the remaining 113 triplets may have immunogenic potential. An accompanying article addresses the relative immunogenicity of polymorphic triplets on class I HLA molecules [58].

Determination of HLA Class I Compatibility at the Amino Acid Triplet Level

HLAMatchmaker applies two principles: (1) each HLA antigen represents a distinct string of polymorphic triplets as potential immunogens that can induce specific alloantibodies; and (2) sensitized patients do not have alloantibodies against triplets present on their own HLA molecules. The algorithm assesses donor-recipient compatibility through intralocus and interlocus comparisons, and determines what triplets on mismatched HLA molecules are different or shared between donor and patient. This analysis considers each donor HLA antigen mismatch towards the entire HLA-A, HLA-B, HLA-C phenotype of the recipient.

As an example, a mismatch between HLA-B18 (donor) and HLA-B7 (recipient) can be characterized by triplet differences in 12 sequence positions: 9, 45, 66, 70, 74, 131, 151, 156, 163, 171, 177, and 180 (Figure 1a). The structural nature of an HLA mismatch is greatly influenced by the recipient's own HLA antigens. For instance, an HLA-B18 mismatch for HLA-B37 represents triplet differences in six sequence positions: 62, 76, 80, 82, 156, and 171, and this triplet mismatch pattern is very different than that seen for HLA-B7 (Figure 1b). For a recipient with the HLA-B7,B37 phenotype, a HLA-B18 mismatch would represent only two triplets, b156L and b171H, whereas all the other triplets of HLA-B18 are shared with HLA-B7 and/or HLA-B37 (Figure 1c). This approach of intralocus triplet sharing is similar to the concept of CREG (or public epitope) matching except that this algorithm considers HLA compatibility in more precise structural detail.

Of course each patient types for HLA-A (and HLA-C) antigens, and HLAMatchmaker incorporates interlocus comparisons of triplet sharing. In this example, the HLA-B7,B37 recipient types also as HLA-A33. Figure 1d reveals that HLA-A33 has the same two triplets, 156L and 171H, in identical positions as the two triplets of HLA-B18 that are different from HLA-B7,37.

FIGURE 1 Example of human leukocyte antigen (HLA) matching at the triplet level.

	Position	9	12	14	17	41	45	58	62	66	70	74	76	80	82	90	105	107	127	131	142	144	147	149	151	155	158	163	166	171	177	180	184	186	193	199	207	246	248	253
a																																								
Recipient	HLA-B7	Y	sV	R	gR	A	Eq	G	Re	dy	aGa	D	Es	dN	Rg	A	P	G	N	R	I	Klr	W	aAr	aRe	R	A	E	Ew	Y	Dk	E	dP	K	Pi	A	G	A	V	E
Donor	HLA-B18	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Es	dN	Rg	A	P	G	N	S	I	Klr	W	aAr	aRy	L	A	T	Ew	H	Et	Q	dP	K	Pi	A	G	A	V	E
b																																								
Recipient	HLA-B37	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Ed	rTl	Ilr	A	P	G	N	S	I	Klr	W	aAr	aRv	D	A	T	Ew	Y	Et	Q	dP	K	Pi	A	G	A	V	E
Donor	HLA-B18	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Es	dN	Rg	A	P	G	N	S	I	Klr	W	aAr	aRy	L	A	T	Ew	H	Et	Q	dP	K	Pi	A	G	A	V	E
c																																								
Recipient	HLA-B7	Y	sV	R	gR	A	Ee	G	Re	dy	aGa	D	Es	dN	Rg	A	P	G	N	R	I	Klr	W	aAr	aRe	R	A	E	Ew	Y	Dk	E	dP	K	Pi	A	G	A	V	E
Recipient	HLA-B37	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Ed	rTl	Ilr	A	P	G	N	S	I	Klr	W	aAr	aRv	D	A	T	Ew	Y	Et	Q	dP	K	Pi	A	G	A	V	E
Donor	HLA-B18	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Es	dN	Rg	A	P	G	N	S	I	Klr	W	aAr	aRy	L	A	T	Ew	H	Et	Q	dP	K	Pi	A	G	A	V	E
d																																								
Recipient	HLA-A33	Y	sV	R	gR	A	Me	G	Re	rNr	aRa	ID	Vd	gTL	Rg	A	S	G	N	R	I	Klr	W	aAr	aRr	L	A	T	Ew	H	Et	Q	dP	R	Av	A	S	S	V	Q
Recipient	HLA-B7	Y	sV	R	gR	A	Ee	G	Re	dy	aGa	D	Es	dN	Rg	A	P	G	N	R	I	Klr	W	aAr	aRe	R	A	E	Ew	Y	Dk	E	dP	K	Pi	A	G	A	V	E
Recipient	HLA-B37	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Ed	rTl	Ilr	A	P	G	N	S	I	Klr	W	aAr	aRv	D	A	T	Ew	Y	Et	Q	dP	K	Pi	A	G	A	V	E
Donor	HLA-B18	H	sV	R	gR	A	Te	G	Re	gls	tNt	Y	Es	dN	Rg	A	P	G	N	S	I	Klr	W	aAr	aRy	L	A	T	Ew	H	Et	Q	dP	K	Pi	A	G	A	V	E

TABLE 3 Examples of zero-, one-, and two-triplet mismatches for different HLA phenotypes

	HLA type of patient*	Zero-triplet mismatch	One-triplet mismatch	Two-triplet mismatch
Case 1	A2,A30; B42,B53; Cw4,Cw7	A69, B8, B35, B55, B56, B59	B51 (b171H)	A68 (a156W,a246Va)
			B54 (b45Grev)	A32 (a107Grl,a246S)
			B67 (b158T)	A74 (a107Grl,a246S)
			B70 (b66qIc)	B7 (b163E,b177Dk)
			B71 (b66qIc)	B38 (b66qIc,b158T)
			B72 (b66qIc)	B39 (b66qIc,b158T)
			B78 (b171H)	B46 (b45Ma,b156W)
				B52 (b66qIs,b171H)
				B58 (b66rNm,b70aSa)
				B64 (b66qIc,b171H)
				B65 (b66qIc,b171H)
				B75 (b45Ma,b66qIs)
				B76 (b45Ma,b156W)
				B77 (b45Ma,b66qIs)
				B78 (b151aRe,b171H)
				B82 (b163dIL,b166Ha)
Case 2	A2,A30; B8,B42; Cw7,-	A69	None	A68 (a156W,a246Va)
				A74 (a107Grl,a246S)
				B54 (b45Gel,b131S)
				B55 (b131S,b151aRe)
				B56 (b131S,b163L)
				B67 (b131S,b158T)
Case 3	A2,A30; B53,B60; Cw3,Cw5	A69, B35, B49, B50, B61	B18 (b171H)	A32 (a107Grl,246S)
			B48 (b45Ile)	A68 (a156W,a246Va)
			B51 (b171H)	A74 (a107Grl,a246S)
			B52 (b171H)	B41 (b156D,B177Dz)
			B59 (b45Ile)	B45 (b156D,b166Ha)
			B72 (b45Ee)	B46 (b45Ma,b156W)
			B75 (b45Ma)	B58 (b66rNo,b70aSa)
			B77 (b45Ma)	B62 (b45Ma,b156W)
			B78 (b171H)	B70 (b45Ee,b66qIc)
				B71 (b45Ee,b66qIc)
Case 4	A3,A11; B8,-;Cw2,-	none	none	B42 (b66qIy,b70aQa)
Case 5	A2,-;B35,-;Cw4,CW8	none	B78 (b171H)	A69 (a66rNv,a70aQs)

*Triplet assignments were made from molecular equivalents of serological antigens. These alleles are listed in Table 1.

means that all polymorphic triplets of HLA-B18 can be found on one or more of the recipient's HLA-A33, HLA-B7, and HLA-B37 antigens. Thus, HLA-B18 must be fully histocompatible to the HLA-A33,X; B7,B37 phenotype, at least from the perspective of antibody-accessible triplets (X can be any A locus antigen).

Table 3 lists five examples of HLA matching at the triplet level; these cases have been selected to illustrate how the HLA phenotype of the patient can influence the number of HLA antigens with zero or few triplet mismatches. Case 1 deals with the HLA-A2,A30; B42,B53; Cw4,Cw7 phenotype that has several antigens that are more common to African-Americans. Six HLA-A, HLA-B antigens are zero-triplet mismatches, namely the cross-reactive HLA-A69, HLA-B35, HLA-B55, and HLA-B56 as well as HLA-B8 and HLA-B59, which are generally not considered to be crossreactive with any antigen in this phenotype. Seven HLA antigens are one-triplet mismatches and 15 HLA antigens are two-triplet mismatches (Table 3) and many of them, but not all (*e.g.*, HLA-B38 and B39), crossreact with patient HLA antigens.

Case 2 illustrates another example: the HLA-A2,A30; B8,B42; Cw7, phenotype differs from Case 1 by only one antigen, HLA-B8 (this antigen is a zero-triplet mismatch for Case 1), and is negative for HLA-Cw4. Only one HLA antigen (HLA-A69) is a zero-triplet mismatch for Case 2. None are one-triplet mismatches and only six HLA antigens (A68, A74, B54, B55, B56, and B67) are two-triplet mismatches (Table 3). Although the HLA phenotypes of Case 1 and Case 2 are very similar, the numbers of HLA antigens with zero or few triplet mismatches are very different.

Case 3 types as HLA-A2,A30; B53,B60; Cw3,Cw5,

and the HLA-A,B phenotype is different from Case 1 in that it has HLA-B60 instead of HLA-B42 (both antigens are members of the B7 CREG). Five HLA antigens are fully matched at the triplet level, nine HLA antigens are one-triplet mismatches, and ten HLA antigens are two-triplet mismatches (Table 3). This group of zero and few triplet mismatches has different HLA antigens than seen for Case 1. For instance, HLA-B49 and HLA-B50 are members of the B5 CREG that includes HLA-B53. These antigens are zero-triplet mismatches for HLA-A2,A30; B53,B60; Cw3,Cw4 (Case 3), but for HLA-A2,A30; B42,B53; Cw4,Cw7 (Case 1) they have four mismatched triplets: b9H, b41T, b45Ke, and b66qIc. One the other hand, the crossreactive HLA-B51 is a one-triplet mismatch for both cases. Thus, depending on the HLA phenotype of the patient, HLA mismatching within crossreactive groups may indicate considerable differences at the triplet level.

For certain HLA phenotypes it is impossible to identify zero-triplet mismatched HLA antigens. Two examples are HLA-A3,A11; B8,-; Cw2,- (Case 4), for which the most compatible HLA-B42 is a two-triplet mismatch and A2,-; B35,-; Cw4,Cw8 for which the best match is the one-triplet mismatched HLA-B78. Homozygosity for the HLA-A and HLA-B loci reduces the numbers of HLA antigens with zero or few triplet mismatches.

The examples in Table 3 illustrate how HLAMatchmaker can be used in the search of compatible donors for highly sensitized patients. The HLA phenotypes of such donors could constitute a combination of HLA antigens from the patient and other HLA antigens that are zero-triplet mismatches. For instance, an HLA-A30,A69; B8,B35 donor would be considered, by conventional criteria, a three-A,B antigen mismatch for an HLA-A2,A30; B42,B53; Cw4,Cw7 recipient (Case 1) but by this matching algorithm this phenotype would have no mismatched triplets and therefore, might be compatible at the HLA-A HLA-B loci.

Considering the numbers and frequencies of HLA antigens with zero-triplet mismatches, the chances of finding a histocompatible donor seem better for Cases 1 and 3 than for Cases 2, 4, and 5. Therefore, many donor searches may have to consider additional HLA antigens, preferably those with one or few triplet mismatches.

The proper selection of a triplet mismatch depends on the antibody specificity repertoire of the sensitized patient. Table 3 illustrates what triplets are involved in the one-triplet and two-triplet HLA antigen mismatches. For instance, in Case 1, the one-triplet mismatched antigens HLA-B51 and HLA-B78 involve the same triplet (b171H), whereas HLA-B70 and HLA-B71 are mismatched for b66qIc. HLA-B64 and HLA-B65 are mismatched for b66qIc and b171H, and HLA- B38 and HLA-B39 are mismatched for b66qIc and b158T. A determination of antibody specificity to polymorphic triplets may identify unacceptable HLA antigens for a given patient. For instance, in Case 1, the presence of an antibody to b66qIc would render the one-triplet mismatches HLA-B70 and HLA-B71 and the two-triplet mismatches HLA-B38, HLA-B39, HLA-B64, HLA-B65 as unacceptable for this patient. An antibody to b171H would rule out HLA-B51, HLA-B64, HLA-B65, and HLA-B78 as potential donor antigens for Case 1. In a clinical setting it is generally not feasible, however, to perform a detailed analysis of antibody specificity patterns of high PRA sera.

Identification of Acceptable HLA Mismatches for High PRA Patients

Nevertheless, there is an additional approach to identify acceptable HLA antigen mismatches for high PRA patients. It considers the HLA types of panel cells that give negative reactions with the patient's serum. Such negative panel cells can be expected to share HLA antigens with the patient, whereas other HLA antigens may contain mismatched triplets that are apparently not recognized by the patient's antibodies. Such triplets would be acceptable even if they are present on other HLA antigens not expressed on the negative panel cells.

Case 6 represents a patient for whom it is difficult to identify HLA antigens with a zero-triplet mismatch. This patient has a 96% PRA with a 50-cell panel and he types as HLA-A3,A29; B18,B47; Cw7,-. The following HLAMatchmaker analysis yields information about HLA antigens with zero- to five-triplet mismatches:

Zero: B61
One: B37
Two: A74 B50
Three: A11 A31 A33 A34 A66 B27 B35 B39 B41 B45 B54 B60 B65 B72 B75
Four: A26 A30 A32 A43 B48 B55 B56 B62 B67 B70 B71 B73 B78
Five: A69 B8 B13 B46 B49 B59 B76

Because only HLA-B61 is a zero-triplet mismatch and HLA-B37 is a one-triplet mismatch, it would be difficult to find a compatible donor for this patient. However, the PRA information may permit the identification of additional HLA antigens with acceptable triplet mismatches. This patient's serum had a 96% PRA with a 50-cell panel and consistently negative reactions were seen with two panel cells with the following HLA types: A3,A33; B27,B64; Cw1,Cw8 and A3,A29; B44,B64; Cw7,-. If a high PRA was due ro HLA-specific antibodies, one could expect that the negative panel cells would share some HLA antigens with the patient and this was apparently the case for this patient. The other HLA antigens on the

TABLE 4 Numbers of triplet mismatches for HLA-C antigens

	HLA type of patient	Cw1	Cw2	Cw3	Cw4	Cw5	Cw6	Cw7	Cw8
Case 1	A2,A30; B42,B53; Cw4,Cw7	1	3	0	—	2	1	—	2
Case 2	A2,A30; B8,B42; Cw7,-	6	9	5	8	9	7	—	5
Case 3	A2,A30; B53,B60; Cw3,Cw5	1	2	—	3	—	4	8	1
Case 4	A3,A11; B8,-;Cw2,-	3	—	2	4	3	1	8	3
Case 5	A2,-; B35,-; Cw4,Cw8	1	3	0	—	1	2	7	—
Case 6	A3,A29; B18,B47; Cw7,-	6	8	5	9	9	7	—	5

The triplet selection was based on the sequences of serologically defined HLA antigens corresponding to: Cw*0102, Cw*0202, Cw*0302, Cw*0401, Cw*0501, Cw*0602, Cw*0701, Cw*0801.

negative panel cells, in this case HLA-A33, HLA-B27, HLA-B44, and HLA-B64, carry unshared triplets but none of them were apparently recognized by the patient's antibodies. This information can be incorporated in the HLAMatchmaker analysis, which yields a new set of HLA antigens with zero/acceptable and few triplet mismatches:

Zero/Acceptable: *A33 B27* **B37** *B44* **B45** **B50** B61 *B64* **B65** **B70** **B71** **B72**

One: A31 A74 B39 B49 B73 B75

Two: A32 B13 B35 B38 B41 B52 B55 B56 B59 B62 B77 B78

Three: A11 A26 A34 A43 A66 B8 B46 B48 B51 B53 B54 B60 B67 B76 B82

Four: A25 A30 B42 B63

Five: A69 B7 B57 B58 B81

As expected, HLA-A33, HLA-B27, HLA-B44, and HLA-B64 are now listed as zero/acceptable triplet mismatches (they are in italic font) because this patient had no antibodies against any triplet on these antigens. HLAMatchmaker identified seven additional HLA antigens (they are in bold and underlined) that became acceptable mismatches because their triplets were not recognized by patient's antibodies. Five HLA antigens became one-triplet mismatches for this patient. With this expanded list of HLA antigens with zero-triplet mismatches, a compatible donor might more readily found for this patient.

HLA mismatch acceptability may also be assessed with information about the immunogenicity of polymorphic triplets. During recent years, the concept has emerged that highly sensitized patients produce a limited repertoire of alloantibodies specific for the more common private and public HLA epitopes [17, 19, 24]. Although most highly sensitized patients have been exposed to many mismatched triplets, their antibody reactivity patterns reveal specificity to a relatively small number of immunogenic triplets, whereas other triplets do not induce an antibody response and, therefore, must be non-immunogenic for the patient. The generation and application of information about immunogenicity of triplets will be addressed in another report [58].

HLA-C Mismatching at the Triplet Level

The degree of HLA class I histocompatibility generally addresses the antigens encoded by the HLA-A and HLA-B loci. HLA-C is largely ignored and, from a matching perspective, this locus has remained an enigma. The serologic polymorphism of HLA-C is poorly defined and HLA-C molecules are expressed on the cell surface at much lower levels than HLA-A and HLA-B molecules [59]. HLA-C antigens appear not very immunogenic [60] and they do not seem to play a major role in the selection of compatible platelet donors for refractory thrombocytopenic patients [61]. Molecular typing has found that HLA-C alleles are more closely related to each other. In particular, the helix of the α1 domain of HLA-C molecules is unusually conserved, whereas the α2 domain is similar to that of HLA-B [62]. The serologically defined HLA-C antigens exhibit less amino acid polymorphism than HLA-A and HLA-B antigens, and this is reflected by the lower numbers of polymorphic triplets in fewer positions in the HLA-C sequences (Table 1).

HLAMatchmaker provides an opportunity to assess HLA-C antigen compatibility at the triplet level. Table 4 illustrates the results of six cases described above, including the five cases listed in Table 3. The numbers of HLA-C triplet mismatches were generally very low for combinations involving all HLA-C antigens except HLA-Cw7, which has four unique triplets. Incompatibility for HLA-Cw7 involves a higher number of mismatched triplets (6–8 triplets) than incompatibility for the other HLA-C antigens (0–3 triplets). Conversely, as Case 2 and Case 6 illustrate, the numbers of HLA-C triplet mismatches are highest for recipients who are homozygous for HLA-Cw7.

These findings provide some insight why, in many cases, HLA-C incompatibility does not seem to play a

major role in humoral HLA-specific alloimmunization. Often enough, an incompatible HLA-C antigen represents a zero or a few triplet mismatch. The exception is HLA-Cw7 (*i.e.*, both HLA-C*0701 and HLA-C*0702) for which the polymorphic triplet repertoire is different than for the other HLA-C antigens. This information is based on serologically defined HLA-C antigens. Molecular typing for HLA-C will permit a more precise assessment of HLA-C compatibility at the triplet level.

DISCUSSION

HLAMatchmaker is an easy to use computer program that determines donor HLA class I acceptability for highly sensitized patients, including kidney and heart transplant candidates, and for refractory thrombocytopenic patients requiring HLA-compatible platelet transfusions. This algorithm permits a determination of the structural basis of an HLA antigen mismatch and utilizes intralocus and interlocus comparisons of strings of amino acid triplets on antibody-accessible sites of HLA class I molecules. Considering the large repertoire of polymorphic triplets on class I HLA molecules, the surprising finding was made that certain HLA antigens, by conventional criteria, are mismatched for a given HLA type, but turn out to be fully compatible at the triplet level. This concept has clinical relevance because HLAMatchmaker can identify acceptable HLA mismatches for sensitized patients and appears to be useful predictor of a cross-match result [63, 64]. Moreover, recent studies have reported that in cadaver kidney transplantation, the zero-HLA-DR mismatched allografts with HLA-A,B mismatches, but no mismatched triplets, have the same survival rates as the zero-HLA-A, B, DR mismatches [65].

This matching algorithm applies the concept that each HLA antigen has multiple epitopes that can elicit specific alloantibodies. These antigenic determinants have been serologically defined as private and public (or CREG) epitopes [25], and many of them correspond to distinct amino acid residues or sequences in HLA molecules. The so-called epitope maps of the HLA-A2 and HLA-B7 CREGs are examples of the structural basis of private and public determinants [32, 33]. However, the information about the total repertoire of serologically defined HLA epitopes remains incomplete and this makes it difficult to apply HLA epitope-based matching strategies for identifying suitable donors for highly sensitized patients.

HLAMatchmaker addresses the total spectrum of antibody-accessible amino acid sequence polymorphisms as critical components of potentially immunogenic epitopes. It considers a linear sequence of three amino acids as a minimal requirement for assessing HLA compatibility at the molecular level. Matching is assessed by determining whether or not a triplet in a given position of a mismatched HLA antigen is also found in the same position in any of the patient's own HLA-A, HLA-B, HLA-C molecules. A shared triplet in the same position on a mismatched HLA antigen cannot elicit a specific antibody response in the patient. This hypothesis has been verified experimentally in an accompanying article [58].

Why triplets? This algorithm considers the structural basis of the interaction between an antibody and a protein antigen. The antibody-binding sites of immunoglobulin molecules comprise six hypervariable loops that make contact with protein antigen [66, 67]. They are referred to as complementarity determining regions (CDR); three are on the heavy chain (CDR-H1, CDR-H2, and CDR-H3) and three are on the light chain (CDR-L1, CDR-L2, and CDR-L3). CDR-H3 has the highest sequence variability and conformational freedom [68], and this loop seems to play a primary role in the antibody specificity while the others enhance the specificity and the strength of binding to the antigen [69]. Three-dimensional structures of antigen-antibody complexes have revealed that the contact area between antibody and antigen is about 700 to 800 square angstroms and it involves about 15–22 pairs of amino acid residues [69–71]. It should be noted that the surface of the HLA molecule seen from above the peptide-binding region and the alpha helices is about 750 square angstroms [48]. With six CDRs on antibody and 15-22 amino acids on antigen as contact sites, one can estimate an average of three amino acids binding to each CDR. There is evidence that sequences of three residues can be recognized by certain antibodies with low affinity [72, 73]. For longer peptides of 4 to 8 residues that bind to an antibody, the replacement of each residue of the peptide by other amino acids will indicate that three contact residues are often essential for binding, whereas the other residues may be replaceable by virtually any amino acid [68, 74]. Thus, in the case of an HLA-specific antibody, one CDR may play a primary role in that it recognizes and binds the polymorphic triplet while the other CDRs interact with other sites on the HLA molecule. Such sites may have monomorphic and polymorphic residues. Site-directed mutagenesis studies have reported that amino acid substitutions in sequence positions distant from the epitope may influence the binding between HLA antigen and antibody [40, 75].

The selection of triplets for matching purposes does not imply that the structural basis of an epitope always involves exactly three amino acids. Many triplets have only one or two polymorphic residues, and some epitopes

might be defined by four or five polymorphic residues in adjacent positions. A typical example are the five amino acid sequence in positions 79–83 that are recognized by Bw4- and Bw6-specific antibodies. Serologic studies have also described a heterogeneity among the specificity patterns of Bw4-associated antibodies suggesting possible subtypes [42, 76, 77]. The application of triplet matching within the 79–83 sequence incorporates this Bw4 heterogeneity. A full mismatch for Bw4 is represented by two mismatched triplets and a partial Bw4 mismatch is indicated by a one-triplet mismatch in the 79–83 sequence. Triplets are also used to incorporate possible epitope heterogeneity in the 149–152 sequence, which has four polymorphic residues in adjacent positions.

For most patients, HLAMatchmaker can identify mismatched HLA antigens that are fully compatible at the triplet level. Many antigens crossreact with the HLA antigens of the patient and this finding is in accordance with the concept of CREG matching in donor selection strategies [21, 78, 79]. Often enough, the program identifies other cross-reacting antigens that are incompatible at the triplet level and should perhaps be avoided for matching purposes. Serologically based CREG matching strategies emphasize public epitopes over private epitopes. Depending on the HLA phenotype of the patient, certain private epitopes have considerable immunogenic potential and mismatching for them might be detrimental. This may explain why the beneficial effect of CREG matching on kidney transplant outcome remains controversial [78–83]. HLAMatchmaker permits a fine tuning of the CREG matching algorithm because it considers the structural organization of public and private epitopes.

The identification of HLA antigens with zero-triplet mismatches is greatly influenced by the patient's HLA phenotype. In several cases, especially if the HLA phenotype reveals homozygosity or has closely cross-reacting antigens, there are no or very few HLA antigens with zero-triplet mismatches. Acceptable HLA antigen mismatches can be identified from HLA-typed panel cells that give negative reactions with patient's serum [84]. It should be emphasized that such negative reactions are obtained with the most sensitive screening technique and that they can be reproduced with several serum samples, especially if the PRA is high. A negative reaction means that patient's antibodies do not recognize any triplet on the mismatched HLA antigen(s) of a given panel cell and this information can be incorporated in HLAMatchmaker for the identification of additional HLA antigens not expressed on the negative panel cells, but can be expected to be acceptable mismatches for the patient. How does one approach the analysis of a serum with a 100% PRA? Obviously, the HLA antigen composition of the panel was such that the patient's antibodies always recognized one or more epitopes on each panel cell. The chances for a negative reaction will increase if panel cells are selected with HLA antigens shared with the patient whereas the other HLA antigens are mismatched for a few triplets. HLAMatchmaker can readily identify such antigens.

Several refinements are needed for an optimal application of the HLAMatchmaker algorithm. The assignment of triplets to HLA antigens may lack precision if the HLA typing information is based solely on serologic methods. DNA-based typing will permit the definition of HLA subtypes and, therefore, more accurate assignments of polymorphic triplets. Many molecular subtypes of serologically defined HLA antigens have different triplets in antibody-accessible positions. In such cases some serologically matched HLA antigens may have incompatible triplets recognized by the patient's antibodies.

Another limitation is the lack of sufficient HLA-C typing information. Serologic typing for HLA-C is often unreliable and only eight HLA-C antigens are considered in the version of HLAMatchmaker described in this report. Molecular methods are now routinely available for HLA-C typing and this permits a more detailed assessment of HLA class I comparibility at the triplet level. The HLAMatchmaker algorithm can also be expanded to class II HLA antigens encoded by the DR and DQ loci.

Although HLAMatchmaker can be used as a crossmatch predictor algorithm to identify potential donors for sensitized patients, it may also offer a new approach of optimizing donor-recipient HLA compatibility with the goal of preventing or reducing antibody-mediated rejection of organ transplants. In a recent review, McKenna *et al.* [85] has emphasized the importance of HLA-specific antibody responses in transplant rejection. Any matching strategy for controlling humoral rejection should consider the overall repertoire of structurally defined epitopes and their immunogenicity relevant to antibody formation. Indeed, a recent study has reported that HLA matching at the triplet level has a beneficial effect on kidney transplant outcome [65].

Various versions of HLAMatchmaker can be downloaded free of charge from the website of the Transplantation Pathology Internet Service of the University of Pittsburgh Medical Center (http://www.tpis.upmc.edu).

ACKNOWLEDGMENTS

This study was supported by Grant DK-52803 from the National Institutes of Health, and by NIH R01grant DK-52803 from the National Institute for Digestive and Kidney Diseases.

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